REMARKS

In the Advisory Action dated October 16, 2003, Claims 45-62 are pending and under examination. Claims 45-62 have been rejected under 35 U.S.C. § 112, first paragraph as allegedly lacking enabling support.

This response addresses each of the Examiner's rejections and objections. Applicants therefore respectfully submit that the present application is in condition for allowance. Favorable consideration of all pending claims is therefore respectfully requested.

The Examiner has maintained the rejection of Claims 45-62 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enabling support. Specifically, the Examiner acknowledges that the techniques that are required to accomplish each of the nuclear transfer steps vary slightly between species. However, the Examiner alleges that these slight variations affect the success or failure of the cloning protocol. The Examiner states that the Declaration of July 12, 2003 executed by Dr. Morrison has not been considered timely filed. Therefore, the Examiner has considered the Declaration only in the relevant part (i.e., paragraphs 9-11) so as to overcome the rejection of Claims 57 and 62 under 35 U.S.C. §112, second paragraph and to obviate certain objections to the specification. The Examiner states that the remainder of the Declaration has not been considered as it allegedly presents new evidence.

In the first instance, Applicants now respectfully resubmit the Declaration (Exhibit 1) and other evidence not fully considered by the Examiner which are attached hereto as Declaration Exhibits A-C and Exhibits 2-4, in connection with the request for continued examination under 37 C.F.R. §1.114. Accordingly, the finality of the previous official action should be withdrawn and the Declaration and other evidence enclosed herewith should be fully considered.

Applicants observe that the present invention discloses that the source of neural stem cells used in the nuclear transfer techniques can be derived from any animal species that has a nervous system, e.g., murine, bovine, ovine, porcine, equine, feline, simian, endangered species, live stocks or from marsupials including kangaroos, wombats. See the specification, at page 11, lines 10-13. Applicants also observe that the specification specifically teaches nuclear transfer experiments in rats, in Examples 12 and 13, at pages 30-32, for example. In addition, Applicants observe that Exhibits 1B and 1C demonstrate that successful nuclear transfer experiments in rat, mouse and bovine models, involve the same technical approaches as illustrated in Examples 1-5 of the present specification. Moreover, Dr. Morrison confirms that the nuclear transfer procedures are the same in all three species. See Declaration, paragraph 14. Most notably, Dr. Morrison testifies that one skilled in the art who can practice nuclear transfer techniques in one species can, with some adjustment to protocol, practice the same techniques in another species. See Declaration, paragraph 14. Dr. Morrison also confirms that the results outlined in Exhibits B and C demonstrate that the description and enablement of the technology in one mammalian species (e.g., rat) by the present invention serves as a validation of the approach in all other (animal) species. See the Declaration, paragraph 15.

Accordingly, Applicants submit that in view of the teachings of the specification and the Declaration together with the evidence presented in Exhibits 1B and 1C, the features of the present invention are readily applicable to all animal species.

The Examiner also alleges that no cloned rat has been reported as of the filing date of the present application. The Examiner further alleges that there is no support for the statement that difficulties encountered in cloning various species could be overcome by routine experimentation or by transferring reconstructed embryos, without first culturing them, to recipient animals.

In response, Applicants direct the Examiner's attention to a publication by Hirabayashi et al., enclosed herewith as Exhibit 2, which outlines the problems inherent in the *in vitro* manipulation of rat oocytes and embryos. Specifically, Hirabayashi et al. indicate that the failure of nuclear transferred rat embryos to develop into full-term offspring is due to microinjection of cell nuclei into oocytes that have spontaneously activated (see Page 35, paragraph 2 of Hirabayashi et al). As indicated by Hirabayashi et al. and according to Applicants' own findings, the reported difficulties in adapting nuclear transfer procedures to the rat is due to the spontaneous activation of the rat oocyte *in vitro*, which reduces the efficiency with which donor nuclei can be reprogrammed and the subsequent development to the morula/blastocyt.

Applicants submit that further optimization of the rat nuclear transfer procedures is required to overcome this propensity, such as using an agent to block the spontaneous activation (see Josefsberg et al., enclosed herewith as Exhibit 4). Applicants submit that, despite these perceived inefficiencies, Applicants have for the first time demonstrated that NSC nuclei are capable of directing rat embryo development into the morula stage (see the Declaration, paragraph 14).

Regarding the transfer of reconstructed embryos directly to recipient animals as opposed to culturing them *in vitro* for a period before transfer, Applicants respectfully direct the Examiner's attention to a publication by Papaioannou et al. (abstract enclosed herewith as Exhibit 3) which describes the use of the mouse oviduct as an improved culture environment for early mouse embryos (see the abstract of Papaioannou et al.). Applicants submit that this is a standard approach used by embryologists to overcome developmental problems that may be introduced by *in vitro* culture. Applicants submit that this approach in animal cloning will only

address the efficacy for animal embryo development between the *in vitro* culture conditions and the *in vivo* culture system.

In addition, Applicants have added Claims 67-70 to further delineate the embodiments of the present invention. Support for Claims 67-70 can be found throughout the specification, on page 11, lines 10-13 and in Examples 12 and 13, at pages 30-32, for example. Support for Claims 67-70 can be also found in the Declaration, paragraphs 12-15 and Exhibits 1B and 1C. No new matter is added.

Accordingly, Applicants submit that based on the teachings of the present invention, together with the Declaration, data shown in Exhibits 1B-1C and the teaching by the references in Exhibits 2-4, the present invention provides sufficient guidance for one skilled in the art to practice the features of the present invention in rat and all other animal species. Although one skilled in the art may have to make minor modifications for a successful protocol in a particular species, such minor modification, in view of the foregoing, would not require undue experimentation. Therefore, the rejection of Claims 45-62 under 35 U.S.C. § 112, first paragraph, is overcome and withdrawal thereof is respectfully requested.

In view of the foregoing amendments and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,

Peter I. Bernstein
Registration No. 43,497

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Encls.: Exhibits 1(A-C)
Exhibits 2-4

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Benjamin Roderick Morrison, et al.	**Examiner:**	A.M. Falk
Serial No.:	09/732,520	**Art Unit:**	1632
Filed:	December 7, 2000	**Docket:**	14390

For: LONG-TERM CELL CULTURE COMPOSITIONS AND GENETICALLY MODIFIED ANIMALS DERIVED THEREFROM

Commissioner for Patents
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Sir:

I, John Roderick Morrison, hereby declare as follows:

1. I am one of the co-inventors named in the above-identified application ("the '520 application).

2. (Education) I hold a Bachelor of Science (B.S.) Degree in Biochemistry and a Doctorate Degree in Biochemistry.

3. (Employment history) I was employed as Senior Scientist at Monash Institute of Reproduction and Development. I am currently employed by CopyRat Pty Ltd.

4. I have worked in the field of Reproductive Biology since 1997.

5. I have authored 10 publications in the field of reproductive biology, cloning and the generation of genetically modified animals.

6. A true and correct copy of my curriculum vitae is attached hereto as Exhibit A.

7. I have read the Final Action dated May 12, 2003, issued in respect of the '520 application. I have been asked to review and comment on issues raised by the Examiner in the Final Action.

8. The Examiner is of the opinion that the amendment filed February 19, 2003 (Paper No. 15) introduces new matter into the disclosure. Specifically, the Examiner is of the opinion that there has been no evidence submitted to verify that rats were used in the experiments of Example 12.

9. As one of the co-inventors named in the '520 application, I confirm that rats were used in the experiments of Example 12. Additional rat data in support of the findings outlined in Example 13 are enclosed herewith as Exhibit B, which describes the embryonic development of rat neural stem cells prepared as per Examples 1-5 of the '520 application and subject to nuclear transfer procedures outlined in Example 12 of the '520 application.

10. With respect to the disclosure of "transfected embryonic fibroblast" in the first column heading in the table on page 32 of the specification, the Examiner has maintained the objection that there is no guidance regarding what was used to transfect the fibroblasts. Specifically, the Examiner is of the opinion that that there is nothing in the specification to suggest that the transfected fibroblast in Example 13 carried the *lacZ* gene.

11. As one of the co-inventors named in the '520 application, I confirm that the *lacZ* gene was used in the transfection experiments referred to in Example 13.

12. In the Final Action, the Examiner states that no support is offered for the Applicant's assertion that procedures used in the rat can readily be used in other species. The Examiner further states that no support is offered for the Applicant's assertion that the

difficulties encountered in cloning various species could be overcome by routine experimentation.

13. I enclose herewith Exhibit C, which describes the formation of bovine embryos following nuclear transfer experiments on bovine neural stem cells.

14. The methodologies and results described in Exhibit C clearly illustrate that the method of isolating neural stem cells and the use of neural stem cells in nuclear transfer experiments in the bovine involve the same basic steps as those used in both the rat and mouse described in Exhibit B. For example, the nuclear transfer procedures described in all three species involve oocyte enucleation; introduction of the neural stem cell nucleus; activation of the oocyte to initiate embryonic development; and, culture *in vitro* to the morula/blastocyst stage. Whilst there is a requirement to adjust the techniques used in each species and differences in development outcomes are seen between species the basic approaches are the same. One skilled in the art in one species could, with some adjustments to protocol, practice the same art in another species, as was the case in Exhibits B and C.

15. In sum, it is my considered scientific opinion that the aforementioned results outlined in Exhibits B and C clearly demonstrate that the description and enablement of the technology in one mammalian species (rat) by the present invention, serves as validation of the approach in all other species.

16. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that those statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and

that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: _____ Dated: July 12, 2003

EXHIBIT A

CURRICULUM VITAE
JOHN RODERICK MORRISON PHD

Current Address: CopyRat Pty Ltd
27-31 Wright St.,
Clayton 3168. Australia.

Telephone: 6- 3-9543-1175
Fax: 61-3-9594 7111
email: jmorrison@copyrat.com.au

Date of Birth: June 7, 1961

Citizenship: Australian

Current appointment: Chief Scientific Officer – CopyRat Pty Ltd

Academic Qualifications:

1991 Ph.D. (Medicine), The University of Melbourne
1986: BSc (Hons, The University of Melbourne

Awards:

1986-90 Baker Medical Research Institute PhD Scholarship
1991-92 American Heart Association Post-doctoral Scholarship
2003- Honorary Senior Research Fellow – Monash University

Previous Appointments:

2001-02 Senior Scientist, Monash Institute of Reproduction and Development
1997-01 Scientist, Monash Institute of Reproduction and Development
1996-97 Scientist I, MRC, London, UK
Supervisors: James Scott (MRC, UK)/ Edward M. Rubin (Lawrence Berkeley National Laboratory (LBNL), USA)
1992-96 Scientist II, MRC, London, UK
Supervisors: James Scott (MRC, UK)/ Edward M. Rubin (LBNL, USA)
1990-92 Post-doctoral Fellow - UCSD, La Jolla, CA
Supervisor: Ray C. Pittman

Industry Consultations:

1996-7 A genome-wide screen for secretory proteins
Amgen, Inc.
1996-7 Knock-out of the plasminogen activated receptor-2 (PAR 2)
COR Therapeutics, Inc.
1995 Development of artificial serum for embryonic stem cells
LifeTechnologies Inc.

Funds raised:

Academic:			
2001-05	NH&MRC	Control of Reproductive Processes	$8.5M *
1999-01	ARC	Regulation of Morphogenesis in the testis	$180K
1999	SMURF	Nuclear transfer technology in the rat	$165K
1998	Monash IVF	A mouse model of Kennedy disease	$10K
Commercial:			
2002	CopyRat Pty Ltd	2nd round fund raising	$1.5M
2002-04	CopyRat Pty Ltd	ARC Linkage grant	$360K
2001-03	CopyRat Pty Ltd	START grant	$1M
2000	CopyRat Pty Ltd	1st round fund raising	$2M

* discontinued Dec 2002.

Local, national and international profile: invited seminars

1996 Use of 2-D protein gels to identify RNA editing events. Department of Energy – Directors Special Seminar Series.
Berkeley CA.
1997 Apobec-1 knockout mouse. Institute of Reproduction and Development. Melbourne Australia.
1998 From Molecular Biology to Genomics. Department of Urology, Monash Medical Centre.
1998 RNA editing made easy. Department of Biological Sciences, Monash University
1998 Human Genome Project. Department of Physiology Post-graduate Seminar Series.
2001 A mouse model of Kennedy disease. Guy's Hospital, London UK.
2001 Developing rat knockout technology. Hammersmith Hospital London UK.
2001 A mouse model of Kennedy disease. Institute of Psychiatry, King's College, London UK.
2001 A mouse model of Kennedy disease. NINDS (NIH) Bethesda, USA.
2001 A mouse model of Kennedy disease. Department of Biological Sciences, Monash University.
2003 Developing technology supporting knockouts in the rat. Rat Physiological Genomics Meeting, Cold Spring Harbor, NY.

COMMERCIAL EXPERIENCE:

I am a founding scientist and Chief Scientific Officer (CSO) of CopyRat Pty Ltd. This is a spin-out of Monash Institute of Reproduction and Development. The project was initiated in 1998 by a small group of Institute scientists. Initial funding was obtained using a Monash strategic grant. I worked with Robert Klupacs, then COO of Monash Institute, to develop a business plan for "CopyRat" which we presented to a variety of VCs and business angels. In August 2000, two separate groups of investors offered to provide seed capital to develop the CopyRat technology. One group was chosen on the basis of the financial support that they offered. I was immediately installed as CSO, however this only became a full-time position in January 2003 as I also maintained a full-time academic research program.

CopyRat plans to develop novel technology supporting gene targeting in the rat. CopyRat has been officially operating since December 2000. To date total funds raised to support this venture exceeds $5M. 5 patents are currently under submission. CopyRat has licensed a number of complementary technologies or alternately developed its own technology in an effort to carve out a clear and unimpeded product line. The short-term goals for the company are to develop rat models suitable for medical and pharmaceutical research. While CopyRat is primarily a R&D company it is envisaged that primary business will become the generation of models for a broad customer base with high volume sales. The company has developed globally with collaborations, licensing deals and contracts in place or under development in USA, France, Japan and Singapore. CopyRat also has a wholly own subsidiary, IngenKO Pty Ltd, which is a mouse knockout business. Currently IngenKO consists of about 15 scientists developing mouse models for medical research and pharmaceutical research on a fee-for-service basis. While this company has a proven track record in producing mouse models there are significant issues regarding the management of personnel and improving production efficiencies.

Current Role as CSO:

- Manage research team (10 scientists)
- Manage and develop R&D program
- Develop strategic view for CopyRat science and commercial development
- Technology acquisition and development
- To identify IP generated by the research program and to manage the internal IP portfolio

RESEARCH EXPERIENCE

(1997-2002) Monash Institute of Reproduction and Development

Kennedy disease mouse: My group has developed a mouse model of Kennedy disease. This is a world first which has been keenly contested between a number of laboratories. The mouse model will be invaluable for studying the pathological mechanisms leading to Kennedy disease. The model has a number of interesting properties i) there appears to be an apparent sensitivity of this model to testosterone; ii) the model appears to degenerate rapidly under caloric restriction. A paper reporting an initial description of this work was recently published in Human Molecular Genetics.

Regulation of Sertoli cell proliferation: The focus of these studies has been to understand the molecular mechanisms regulating the proliferative phase of the Sertoli cell. This has resulted in the development of novel methods for the isolation of ultra-pure preparations of cells as well as critical insights into the coordination of

factors regulating the proliferative phase. Two papers reporting these findings have recently been accepted for publication a further 3 papers are in preparation.

Follistatin biology: My group has completed the assembly of three follistatin genomic PAC clones representing a full-length gene and two alternate splicing transcripts. The full-length gene is currently being injected for transgene production. Mice are currently being breed onto the FS knockout background to assess the ability of this gene to rescue the ko mouse.

(1992-1997) Studies on the editing of apolipoprotein B RNA

I worked on a number of projects studying the biochemistry of apobec-1, the catalytic component required for the editing of apo-B RNA. I performed the initial enzymatic studies using oocyte extracts expressing apobec-1, which indicated that apobec-1 had cytidine deaminase activity. My most valuable contribution was to develop a mouse model which lacked Apobec-1, the catalytic subunit, required for apoB RNA editing. These studies were performed in Edward Rubin's lab at the Lawrence Berkeley National Laboratory (1994-1997). This project required that I isolate a mouse genomic clone of apobec-1, map the gene, and design and construct a targeting vector suitable for ablation of the gene in embryonic stem cells. Complementary to this work I have also made a transgenic mouse carrying a human P1 artificial chromosome (PAC) clone (140 kb), which should display the human expression pattern of *APOBEC-1*.

(1990-1992) Studies on the transfer of cholesteryl esters from high-density lipoprotein to the plasma membrane of cells and synthetic membranes

This project involved delineating a mechanism of transfer of cholesteryl esters (and neutral molecules in general) from high-density lipoprotein to cell membranes. Kinetic analysis revealed that this was a collision-mediated event which was able to proceed independent of other factors in either the lipoprotein or the cell membrane fractions. The work required a considerable amount of lipid chemistry, including the manufacture of very large uniform unilamella liposomes (a method I developed), chemical assembly of radiolabelled cardiolipin as well as the use of non-degradable tracers in both lipid and protein fractions.

(1986-1990) Determining the ligand binding domain of the high density lipoprotein receptor

I defined the binding domain of apolipoprotein AI (apo-AI) for a putative high-density lipoprotein receptor on the liver. To achieve this I had to first confirm that a binding site on the liver actually existed. Although there were more than one hundred papers on this subject my studies revealed that the reported low affinity/high capacity site did not follow the laws of mass action, required for a bona fide receptor. Subsequently, I described a new site which was of high affinity/low capacity. Using CNBr fragments of apo-AI bound to phospholipid discs, I determined that the receptor-binding domain resided in the carboxyl-terminus.

PUBLICATIONS

Patents:

1) A cellular composition including neural stem cells. (National Phase).
2) Targeting Methods and vectors and uses thereof. PCT filed Sept 2002
3) Generation of a rat neural stem cell with a long-term growth potential. PCT filed Nov 2002
4) A transgenic model for a neurodegenerative disorder. Provisional filed Jan 2002-12-10
5) Methods of generating non-human transgenic animals and cells derived therefrom. Provisional filed Nov 2002.

Research Papers:

1) Roh S, Malakooti N, Morrison JR, Trounson AO, Du ZT, (2003). *Parthenogenetic activation of rat oocytes and their development in vitro Reproduction, Fertility and Development*, 15, 1–6.
2) Roh S, Guo J, Malakooti N, Morrison JR, Trounson AO, Du ZT (2003) *Birth of rats following nuclear exchange at the 2-cell stage*, Zygote, In press.
3) Buzzard JJ, Wreford NG, Morrison JR, (2003) *Thyroid Hormone, Retinoic Acid and Testosterone Suppress Proliferation and Induce Markers of Differentiation in Cultured Rat Sertoli Cell. Endocrinology, In press.*
4) Buzzard JJ, Farnworth PG, de Kretser DM, O'Connor AE, Wreford NG, Morrison J (2003) *Proliferative Phase Sertoli Cells display a developmentally regulated response to Activin In Vitro. Endocrinology* 144: 474–483

5) McManamny P, Chy HS, Finkelstein DI, Crack PJ, Kola I, Cheema SS, Horne MK, Wreford NG, O'Bryan MK, de Kretser DM, Morrison JR (2002). *A mouse model of SBMA.* Human Molecular Genetics 11: 2103-2111.
6) Hickox DM, Gibbs, G, Morrison JR, Sebire K, Edgar K, Keah HH, Alter K, Loveland KL, Heam MTW, de Kretser DM O'Bryan MK, (2002) *Identification of a novel mouse testis-specific member of the phosphotidylethanolamine binding protein family – PEBP2.* Biology of Reproduction. 67: 917-927
7) Buzzard JJ, Wreford NG, Morrison JR (2002) *Dramatic extension of the rat Sertoli cell proliferative phase using recombinant human FSH.* Reproduction 124: 633-641
8) Hayes E., Lacham-Kaplan O., Galea S., Verkuylen A., Pera M., Morrison J.R., Trounson A., (2001). *Nuclear transfer of normal and genetically modified somatic cells in the rat.* Physiol Genomics 5: 193-203.
9) Buzzard J.J., Morrison J.R., O'Bryan M.K., Song Q., and Wreford N.G., (2000). *The developmental expression of thyroid hormone receptors in the rat testis. Evidence for a novel thyroid hormone receptor transcript.* Biol. Reprod., 62: 664-669.
10) Morrison J.R., Pászty C., Stevens M.E., Hughes S.D., Forte T., Scott J., Rubin E.M. (1996) *Apolipoprotein B RNA editing enzyme-deficient mice are viable despite alterations in lipoprotein metabolism.* Proceedings of the National Academy of Sciences, 93: 7154-7159
11) Morrison JR; Silvestre MJ; Pittman RC. (1994) *Cholesteryl ester transfer between high density lipoprotein and phospholipid bilayers.* Journal of Biological Chemistry, 269:13911-8.
12) Bhattacharya S; Navaratnam N; Morrison JR; Scott J; Taylor WR. (1993) *Cytosine nucleoside/nucleotide deaminases and apolipoprotein B mRNA editing.* Trends in Biochemical Sciences, 19:105-6.
13) Navaratnam N; Morrison JR; Bhattacharya S; Patel D; Funahashi T; Giannoni F; Teng BB; Davidson NO; Scott J. (1993) *The p27 catalytic subunit of the apolipoprotein B mRNA editing enzyme is a cytidine deaminase.* Journal of Biological Chemistry, 268:20709-12.
14) Allan CM; Fidge NH; Morrison JR; Kanellos J. *Monoclonal antibodies to human apolipoprotein AI: probing the putative receptor binding domain of apolipoprotein AI.(1993)* Biochemical Journal, 290:449-55.
15) Morrison JR; McPherson GA; Fidge NH. (1992) *Evidence for two sites on rat liver plasma membranes which interact with high density lipoprotein.* Journal of Biological Chemistry, 267:13205-9.
16) Morrison J; Fidge NH; Tozuka M. (1991) *Determination of the structural domain of ApoAI recognized by high density lipoprotein receptors.* Journal of Biological Chemistry 266:18780-5.
17) Vanloo B; Morrison J; Fidge N; Lorent G; Lins L; Brasseur R; Ruysschaert JM; Bacrt J; Rosseneu M. (1991) *Characterization of the discoidal complexes formed between apoA-I-CNBr fragments and phosphatidylcholine.* Journal of Lipid Research, 32:1253-64.
18) Tetaz T; Morrison JR; Andreou J; Fidge NH. *Relaxed specificity of endoproteinase Asp-N: this enzyme cleaves at peptide bonds N-terminal to glutamate as well as aspartate and cysteic acid residues(*1990). Biochemistry International, 22:561-6.
19) Morrison JR; Fidge NH; Grego B. (1990) *Studies on the formation, separation, and characterization of cyanogen bromide fragments of human AI apolipoprotein.* Analytical Biochemistry 186:145-52.
20) Fidge N; Morrison J; Nugent T; Tozuka M. (1989) *Monoclonal antibodies to human A-I apolipoprotein and characterisation of cyanogen bromide fragments of apoA-I.* Biochimica et Biophysica Acta, 1003:84-90.
21) Tilley L; Sawyer WH; Morrison JR; Fidge NH. (1988) *Rotational diffusion of human lipoproteins and their receptors as determined by time-resolved phosphorescence anisotropy.* Journal of Biological Chemistry, 263:17541-7.
22) Simpson RJ; Smith JA; Moritz RL; O'Hare MJ; Rudland PS; Morrison JR; Lloyd CJ; Grego B; Burgess AW; Nice EC (1985). *Rat epidermal growth factor: complete amino acid sequence. Homology with the corresponding murine and human proteins; isolation of a form truncated at both ends with full in vitro biological activity.* European Journal of Biochemistry, 153:629-37.

Reviews:

1) Shyr-Yeu Lin[1,2], John R. Morrison[1], David J. Phillips[1] and David M. de Kretser[1,*] (2003) *The regulation of ovarian function by the TGF-β superfamily and follistatin* Reproduction, In press.
2) Scott J; Navaratnam N; Bhattacharya S; Morrison JR. *The apolipoprotein B messenger RNA editing enzyme.* Current Opinions in Lipidology, 1994, 5:87-93.

Exhibit B

Additional Rat Data

Rat neural stem cells prepared as per Examples 1-5 in the application were utilised in the nuclear transfer procedures described in Example 12 of the application. The reconstructed eggs were cultured to the morula/blastocyst stage to determine the success of the nuclear transfer procedures. The results obtained are summarised in the following table:

Cell type	n	PN	2-cell	4-cell	Morula	Blastocysts
RFNSC**	158	109	72	11	5	0

** The data were based on 5 experiments.

PN represents the number of reconstructed embryos showing successful pronuclei formation.

RFNSC represent wild-type rat foetal neural stem cells;

The successful development of the reconstructed embryos beyond the 2-cell stage (at which point in the rat development becomes dependent on the embryonic genome as opposed to nutrients and proteins derived from the maternal oocyte) to the morula stage demonstrates that reprogramming of the neural stem cell nuclei enabled the expression of the relevant genes required to direct embryonic development. In the rat, therefore, neural stem cells represent a viable source of donor nuclei for nuclear transfer experiments.

Mouse Data

Mouse foetal neural stem cells were isolated and cultured essentially as described in Examples 1-5 of the application with the age and developmental time points of the foetus used adjusted accordingly. The isolation of mouse oocytes and subsequent nuclear transfer procedures are further described below:

Superovulation and Oocyte Collection of Mice

Four week old F1 female mice (CBA x C57B) were superovulated by intraperitoneal injection of 10 IU PMSG at 8:00 pm followed 48 hours later by 10 IU hCG. Treated female mice were killed by cervical dislocation, about 13 hours after hCG injection and the oviducts removed and transferred into a petri dish containing 3 ml HTF-HEPES medium supplemented with 300 IU/ml hyaluronidase. The oviduct ampullae were opened with fine watch-maker forceps and 30-gauge needle and the cumulus enclosed oocytes released. The dish was then placed on a warm plate at 37°C for 3-5 minute and as soon as the oocytes had separated

from cumulus cells they were recovered with a fine "hand-pulled" glass pipette and washed in two changes of embryo handling medium (HTF-Hepes). The oocytes were then placed in lots of approximately 30-40 in drop with 10 µl handling medium overlaid with mineral oil on the manipulation chamber.

Enucleation, Nucleus Injection, Activation and Culture

The enucleation of mouse oocytes was carried out 13-15 hrs after hCG injection by slitting the zona pellucida in the region of the cytoplasmic bulge using a microneedle with subsequent squashing of the metaphase plate through the slit with the holding pipette in Hepes-Buffer. The enucleated eggs were then placed in HTF embryo culture medium for 1 h before nuclear injection. Nuclei of mouse neural stem cell were injected into enucleated oocytes using pipettes drawn to an approximate inner diameter of 5 µm, which were back-loaded with mercury and coated with 5% polyvinylpyrrolidone (PVP) immediately before use. The pipette containing an isolated nucleus was put through the slit in the zona pellucida and advanced between half and three-quarters of the way through the oocyte. Piczoelectric actuation was used to break the membrane and the nucleus was deposited. The reconstructed eggs were then incubated for 10 min in Hepes at room temperature before being placed into the HTF embryo culture medium for 1 hour. Oocytes were then transferred to an activation medium containing Ca_2–free HTF-Hepes; 10 mM $SrCl_2$; and, 5µg/ml cytochalasin B for 6 hours. Following activation the eggs were washed twice with HTF embryo culture medium and cultured in 10mm wells containing 400 µl of culture medium overlaid with paraffin oil at 37°C in 5% CO_2 in air. The formation of a pronucleus was checked the next morning and the development of the eggs was recorded for 6 days. Cumulus cells freshly isolated from oocytes during the denuding (hyaluronidase treatment) step described above were also used as donor nuclei in order to compare the resulting development of the reconstructed eggs in vitro. Cloned mice have previously been reported using cumulus cells as the donor nuclei (Wakayama *et al*, 1998: Nature 394(6691): 369-74).

Results

The *in vitro* development results of the mouse nuclear transfer experiments described above are summarised in the following table:

Cell type	N	PN	2-Cell	4-cell	Morula	Blastocyst
Cumulus	182*	154	135	78	70	12
MFNSC	38**	28	10	4	3	3

*The data were based on three experiments.

** The data were based on 1 experiment.

PN represents the number of reconstructed embryos showing successful pronuclei formation.

MFNSC represent wild-type mouse foetal neural stem cells.

As for the rat above, the successful development of the reconstructed embryos beyond the 2-cell stage (at which point in the mouse development becomes dependent on the embryonic genome as opposed to nutrients and proteins derived from the maternal oocyte) to the morula and blastocyst stages demonstrates that reprogramming of the neural stem cell nuclei enabled the expression of the relevant genes required to direct embryonic development. In addition, although development to the morula stage was higher in embryos reconstructed with cumulus cells than those with neural stem cells, the development of embryos to the blastocyst stage at an equivalent rate in both groups suggests that such nuclei are similar in their ability to be reprogrammed and to direct embryonic development. As discussed above, embryos reconstructed using cumulus cell nuclei have previously resulted in live born pups. In the mouse, therefore, as for the rat, neural stem cells represent a viable source of donor nuclei for nuclear transfer experiments.

Exhibit C

Bovine Data

Bovine fetal neural stem cells were isolated and cultured essentially as described in Examples 1-5 of the application with the age and developmental time points of the fetus used adjusted accordingly. Fetuses were isolated from freshly killed animals at an abattoir in Poowong, Melbourne, Australia. Fetuses with an approximate crown to rump length of 4-5cm were chosen for the isolation of neural stem cells. The isolation of bovine oocytes and subsequent nuclear transfer procedures are further described below:

Collection of Bovine Oocytes

Bovine ovaries were obtained from a local slaughterhouse, transported at 25-30°C to the laboratory and washed in warmed phosphate buffered saline (PBS, Baxter, Australia). Ovarian antral follicles (2-8mm) were aspirated using an 18-gauge needle and collected into Hepes buffered Tissue Culture Medium 199 (TCM199, Gibco BRL/Life Technologies) with heparin (5000iu/ml, Sigma), 2% Fetal Calf Serum (FCS, Gibco/Life Technologies), and amphotericin B (250μg/ml, Sigma). Cumulus oocyte complexes (COC's) showing an even cytoplasm and surrounded by at least three layers of compact cumulus cells were collected from the follicular fluid. COC's were incubated and matured in groups of 25 in a TCM199 medium supplemented with gentamycin sulfate (10mg/ml), L-glutamine (29mg/ml, Sigma), human Chorionic Gonadotrophin (1500IU/ml, Lyppards, Australia) and 15% FCS at 39°C in 5%CO_2 in air, for 20-24 hours.

Preparation of Oocytes for Nuclear Transfer

In order to remove the surrounding cumulus, matured oocytes at 19-21 hours post maturation (hpm) were vortexed in 80μl maturation media and 20μl hyaluronidase (0.1%, Sigma) for 3 minutes in Eppendorf tubes (Quantum Scientific). The oocytes were washed through handling media (Hepes buffered TCM199 with 5% FCS (199HF)) and those at the metaphase II stage (i.e. with the first polar body extruded) were selected for nuclear transfer (NT).

Nuclear Transfer

Bovine oocytes were enucleated at 20-22hpm in handling media containing cytochalasin B (0.25µl/ml, Sigma) by gentle aspiration of the polar body and metaphase plate in a small amount of cytoplasm using a glass pipette (inner diameter: 10-15µm). The cytoplasts were transferred into TCM199 with 10% FCS and incubated at 39°C in 5% CO_2 in air, until microinjection. After mechanical disruption of the cell membranes in 199HF using the injection pipette, neural stem cells were injected directly into the cytoplasts. The reconstructed embryos were transferred back into TCM199 + 10% FCS until activation.

The reconstructed embryos were activated 30 minutes after microinjection. Embryos were activated in 32µm Ca^{2+} ionophore (Sigma) in TCM199 supplemented with Gentamycin sulfate (10mg/ml) and L-glutamine (29mg/ml) for 10 minutes, followed by a 5-hour incubation in 2mM 6-DMAP (Sigma). Embryos were cultured in modified Synthetic Oviductal Fluid (SOF) culture media supplemented with amino acids (Sigma), 5% FCS, myo-inositol (0.05g/10ml, Sigma) and sodium tri citrate (1mg/1ml, Selby Scientific). Embryos were submerged in a Submarine-Incubation-System (SIS). The 4-well plates were gassed in foil bags (Wests Packaging Services) with 5% O_2, 5% CO_2 and 90% N_2 and immersed in 39°C water for up to six days.

Results

The *in vitro* development results of the bovine nuclear transfer experiments described above are summarised in the following table:

Cell type	N	2-Cell	4-cell	8-cell	Morula	Blastocyst
BFNSC*	250	170	138	115	71	54

*The data were based on sixteen experiments.

BFNSC represent wild-type bovine fetal neural stem cells.

As for the rat and mouse above, the successful development of the reconstructed embryos beyond the 8-10 cell stage (at which point in bovine development becomes dependent on the embryonic genome as opposed to nutrients and proteins derived from the maternal oocyte) to the morula and blastocyst stages demonstrates that reprogramming of the neural stem cell nuclei enabled the expression of the relevant genes required to direct embryonic development. In the bovine, therefore, as for the mouse and rat, neural stem cells represent a viable source of donor nuclei for nuclear transfer experiments.

CLONING AND STEM CELLS
Volume 5, Number 1, 2003

Factors Influencing Chromosome Condensation and Development of Cloned Rat Embryos

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ABSTRACT

Factors influencing premature chromosome condensation (PCC) in transferred rat nuclei have been examined. Chromosome condensation of rat cumulus cell nuclei did not occur when the cell nuclei were injected into enucleated rat oocytes. By contrast, chromosome condensation did occur after transfer to enucleated mouse oocytes or intact rat oocytes. In the first serial NT experiment, rat somatic cell nuclei were injected into enucleated mouse oocytes, and the reconstructed oocytes were activated by strontium chloride. From these reconstructed embryos, karyoplasts containing pronucleus-like vesicles were transferred into pronuclear zygote-derived cytoplasts by a DC pulse. Transfer of a total of 340 serial NT zygotes into recipient females, including 206 two-cell embryos, resulted in only seven implantation sites. In the second serial NT experiment, rat somatic cell nuclei were injected into intact rat oocytes; the recipient metaphase-plate was then aspirated under UV light from the NT oocytes in which PCC of injected nuclei was observed. After activation of the NT oocytes, karyoplasts were introduced into zygote-derived cytoplasts. Transfer of a total of 115 serial NT zygotes, including 37 two-cell embryos, resulted in four implantation sites but no live offspring. These results establish a mean of inducing chromosome condensation in rat oocytes and demonstrate that reconstructed rat zygotes can be prepared by serial NT procedures. Developmental competence of these embryos remains to be clarified.

INTRODUCTION

NO CLONED RAT OFFSPRING have been reported following somatic cell nuclear transplantation (NT). Although full-term development of rat embryos reconstructed with embryonic blastomeres has been reported (Kono et al., 1988), recent attempts to produce cloned rats using fetal fibroblast cells (Fitchev et al., 1999) and adult somatic cells (Hayes et al., 2001; Iannaccone et al., 2001; Kato et al., 2001) have not been successful despite successful cloning of the mouse (Wakayama et al., 1998), rabbit (Chesne et al., 2002), cat (Shin et al., 2002), goat (Baguisi et al., 1999), sheep (Wilmut et al., 1997), pig (Onishi et al., 2000), and cow (Kato et al., 1998).

Rat oocytes have been reported to activate spontaneously during *in vitro* culture (Koefer and Schuetz, 1982; Zernicka-Goetz, 1991; Kato et al., 2001). The activated oocytes extruded the second polar body within 60–90 min of culture, but development became arrested again without for-

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mation of a pronucleus (PN). Therefore, the failure of NT rat embryos to develop into full-term offspring (Hitchev et al., 1999; Hayes et al., 2001; Iannaccone et al., 2001; Kato et al., 2001) may be due to microinjecting cell nuclei into oocytes that have already activated spontaneously. Premature chromosome condensation (PCC), which has been considered essential for successful mouse cloning (Wakayama et al., 1998; Wakayama et al., 1999; Ogura et al., 2000b), would not occur even if oocytes with decreased MPF activity were enucleated and received somatic cell nuclei.

Kwon and Kono (1996) first reported serial NT in the mouse. In the procedure, blastomere nuclei were first transferred into enucleated oocytes by fusion and a pronuclear-like structure allowed to form. In the second transfer, karyoplasts containing the PN-like vesicle were transferred into cytoplasts derived from pronuclear-stage zygotes. It is suggested that the donor nuclei are allowed time for reprogramming during the first NT, and that the cytoplasm of pronuclear zygotes supports further development of the second NT embryos. After serial NT of metaphase-arrested fetal fibroblast nuclei, the proportion of stillborn fetuses with severe abnormalities was reduced (Ono et al., 2001). In the present study, cloning of rats has been attempted by combining the serial NT procedure with PCC induction in somatic cell nuclei.

A series of three experiments have been carried out. In Experiment 1, rat or mouse cumulus cell nuclei were injected into enucleated rat or mouse oocytes, and the incidence of PCC in the homogenous or heterologous oocytes was assessed. In Experiment 2, nuclei from rat cumulus and Sertoli cells were injected into enucleated mouse oocytes. After activation by strontium, karyoplasts containing PN-like vesicles were separated from the heterologous NT oocytes and were fused with cytoplasts derived from pronuclear rat zygotes. Next day, the reconstructed zygotes were transferred into pseudopregnant recipient rats. In Experiment 3, nuclei from rat cumulus and Sertoli cells were injected into fresh ovulated rat oocytes. The second metaphase-plate of the ovulated oocytes was then aspirated from the recipient oocytes in which PCC of injected nuclei was observed. After activation, karyoplasts from these oocytes were fused with rat cytoplasts, cultured overnight and transferred into recipient rats.

MATERIALS AND METHODS

Oocytes

The specific-pathogen-free/virus antibody-free rats and mice were housed under controlled lighting (light on 05:00–17:00 h), temperature (23 ± 2°C) and humidity (55 ± 5%), with free access to a laboratory diet (MF; Oriental Yeast, Co., Tokyo, Japan) and filtered water. Mice were superovulated by intraperitoneal injections of 5 IU eCG (Nippon Zenyaku, Co., Fukushima, Japan) and 5 IU hCG (Sankyo, Co., Tokyo, Japan) 48 h apart, and cumulus-oocyte complexes (COCs) were recovered from the oviductal ampullae 14 h post hCG injection with CZB medium (Chatot et al., 1990) containing 0.1% hyaluronidase (Sigma-Aldrich Corp., St. Louis, MO). The denuded oocytes were washed three times with fresh CZB medium and kept in CZB medium at 37°C. Rats were superovulated with 30 IU eCG and 30 IU hCG at 48 h intervals (Hirabayashi et al., 2001), and COCs were recovered 14 h post hCG injection with modified KRB medium (Toyoda and Chang, 1974) containing 0.1% hyaluronidase. The oocytes were then washed and kept in the modified KRB medium at 37°C.

Donor cells

Cumulus cells were dispersed from COCs by 5-min treatment with 0.1% hyaluronidase in modified KRB medium. The modified KRB medium supplemented with 22 mM Hepes but reduced to 5 mM $NaHCO_3$ was defined as Hepes-KRB medium. An aliquot (2 μL) of the cumulus cell suspension was transferred to 10 μL of Hepes-KRB medium supplemented with 12% polyvinyl pyrrolidone (w/v; PVP, 360 kDa; ICN Pharmaceuticals, Inc., Costa Mesa, CA) (hereafter defined as Hepes-KRB/PVP medium), and stored at room temperature for up to 1.5 h before injection.

Sertoli cells were prepared according to the method reported by Ogura et al. (2000a). The donor male rats were autologous F1 hybrid (Sprague-Dawley × Dark-Agouti) at 3 days old. The freshly isolated cell suspension was cultured for 1 week in Dulbecco's modified Eagle's medium containing 3 μg/mL BSA and 1 μg/mL porcine FSH, and was preserved in a commercially available cryoprotectant solution (Cellbanker 2; NZK Biochemicals, Fukushima, Japan).

After being thawed, the Sertoli cells were further cultured for a week and transferred to Hepes-KRB/PVP medium as were the cumulus cells.

Serial nuclear transplantation

The first NT procedure included the nuclear injection of somatic cells into oocytes using a piezo-micromanipulator (Prime Tech, Ibaraki, Japan), as reported by Wakayama et al. (1998). Nuclei of cumulus or Sertoli cells were isolated just before injection with shear stress using a blunt-end pipette 7–10 μm in diameter and piezo-micromanipulator, and then injected.

The second NT procedure included the electrical fusion of karyoplasts derived from the first NT oocytes with cytnplasts derived from pronuclear-stage rat zygotes. The pronuclear zygotes were collected from superovulated Wistar rats (8–13 weeks old, Charles River Japan) and were used after cryopreservation (Hirabayashi et al., 1997). For cytoplast preparation, both female and male pronuclei and polar bodies were removed in Hepes-KRB medium containing 5 μg/mL cytochalasin B (CB, Sigma-Aldrich). Karyoplasts containing PN-like vesicles were placed into the perivitelline space of the cytoplasts, and the karyoplast-cytoplast couplets were fused in the Zimmerman cell fusion medium (Zimmerman and Vienken, 1982), using an electrofusion apparatus (ECM 200, BTX, San Diego, CA), with one direct current pulse at 20V/mm for 20 μsec. The reconstructed zygotes were then cultured in modified KRB medium for 16–17 h at 37°C in 5% CO_2 in air.

Embryo transfer

After the 16–17 h culture, the reconstructed zygotes were washed with the modified KRB medium and were transferred into the oviductal ampullae of the recipient Wistar rats (8–13 weeks old, Charles River Japan) which had been previously mated with vasectomized male rats. The embryo transfer was performed on Day 1 of pseudopregnancy (the day when the vaginal plug was detected). Eight to 21 embryos were transferred per recipient. On Day 21, the fetal development in the recipients was examined by Caesarean section.

Experiment 1

Wistar female rats at 8–13 weeks old and BDF1 female mice at 8 weeks old were purchased from Charles River Japan, Inc., (Kanagawa, Japan), and used as donor animals for oocytes and cumulus cells. Rat and mouse oocytes were enucleated by aspiration of the metaphase-plate with a small volume of surrounding cytoplasm in Hepes-KRB medium and M2 medium (Quinn et al., 1982) containing 2 μg/mL CB respectively. Injection of cumulus cell nuclei was completed within 1.5 h from oocyte collection. Rat oocytes injected with rat or mouse cell nuclei were cultured in modified KRB medium, and mouse oocytes injected with rat and mouse cell nuclei were cultured in CZB medium at 37°C in 5% CO_2 in air. One hour after the injection, the oocytes were stained with 5 μg/mL Hoechst 33342 (Sigma-Aldrich) and were observed for the incidence of PCC under 330–380 nm UV light (Fig. 1).

Experiment 2

Cumulus cells were prepared from 8–13-week-old F1 hybrid rats (Sprague-Dawley × Dark-Agouti, Japan SLC, Inc., Shizuoka, Japan), and Sertoli cells were prepared as described above. These cell nuclei were injected into enucleated mouse oocytes within 1.5 h of the oocyte collection. The heterologous NT oocytes were kept for 1 h in CZB medium, and then activated by 10 mM $SrCl_2$ (Wako Pure Chemical Industries, Co., Osaka, Japan) in Ca^{2+}-free CZB medium for 6 h at 37°C in 5% CO_2 in air. One hour after being removed from the $SrCl_2$-containing medium, karyoplasts were fused with zygote-derived cytoplasts, and the reconstructed zygotes were cultured until transferred.

Experiment 3

Cumulus and Sertoli cells from the F1 hybrid rats were injected into fresh ovulated oocytes from 4–5-week-old Wistar rats (Charles River Japan) within 1 h of the oocyte collection. Throughout the process of oocyte recovery to enucleation, 20 μg/mL *N*-acetyl-leucyl-leucyl-norleucynal (ALLN, Sigma-Aldrich) was supplemented to the modified KRB or Hepes-KRB medium, to arrest cell cycle of the oocytes at metaphase. One hour after NT, the metaphase-plate of the recipient oocytes was removed by aspiration after staining with Hoechest 33342 under fluorescence microscopy (10 sec for upper limit of UV irradiation per oocyte). Then, the

oocytes were activated for 6 h with 1.25 mM $SrCl_2$ as reported previously (Kato et al., 2001). One hour after being removed from the $SrCl_2$ medium, karyoplasts were fused with zygote-derived cytoplasts, and the reconstructed zygotes were cultured until transferred.

Statistical analysis

All experiments were repeated at least four times. The data were analyzed by Fisher's exact probability test using the StatView program (Abacus Concepts, Inc., Berkeley, CA). A value of $p < 0.05$ was chosen as an indication of statistical significance.

RESULTS

Incidence of PCC in rat or mouse cumulus cell nuclei microinjected into enucleated rat or mouse oocytes (Experiment 1) is summarized in Table 1. Enucleated rat oocytes did not support the PCC of rat and mouse cell nuclei, while more than 90% of enucleated mouse oocytes were able to induce PCC in both rat and mouse nuclei injected (Fig. 1A). In the PCC-negative oocytes, the size and shape of injected nuclei did not change, and the nuclear envelope seemed to remain intact (Fig. 1B).

Therefore in Experiment 2, the potential of enucleated mouse oocytes to support PCC of rat cell nuclei was used for the serial NT procedure, as shown in Table 2. When the cumulus and Sertoli cells were injected into enucleated mouse oocytes, 69 and 61% of the oocytes survived the nuclear injection, and 66 and 56% of the surviving oocytes carried two PN-like vesicles, respectively. Similar proportions of the karyoplast-cytoplast couplets were fused by a DC pulse (78% and 95%, respectively) and cleaved to the two-cell stage (59% and 56%, respectively) in the groups of cumulus and Sertoli cell injection. Finally, 167 and 174 reconstructed zygotes, including 103 and 103 two-cell stage embryos (Fig. 2A), were transferred into 9 and 10 recipients, respectively, resulting in five (3%) and two (1%) implantation sites but no live offspring.

In Experiment 3, rat oocytes before enucleation were used as host cytoplasm to support PCC of injected rat cell nuclei, as shown in Table 3. Survival rates of the intact rat oocytes injected with cumulus and Sertoli cell nuclei were similar (72% and 73%, respectively), and PCC of the injected nuclei was found in 60% and 47% of the surviving oocytes, respectively. From the NT oocytes carrying PCC, the recipient metaphase-plate was removed under UV light at the success rates of 83–85%. In the group receiving cumulus cell nuclei, 79% of the NT oocytes survived strontium treatment, and 58 and 17% of the surviving oocytes carried one and two PN-like vesicles, respectively. Such vesicles isolated as karyoplasts were placed in the perivitelline space of enucleated zygotes at a success rate of 87%, and 65% of these pairs fused. Transfer of 69 reconstructed zygotes including 25 two-cell stage embryos into six recipients resulted in four implantation sites without chorioallantoic placenta (6%, Fig. 2B). In the group receiving Sertoli cell nuclei, 80% of the NT oocytes survived strontium treatment, and 55 and 10% of the surviving oocytes carried one and two PN-like vesicles, respectively. All the karyoplasts were successfully placed in the perivitelline space of enucleated zygotes, and 77% were fused. A total of 46 reconstructed zygotes, including 12 two-cell stage embryos, were transferred into four recipients, but no implantation sites and live offspring were detected.

DISCUSSION

The present experiments have demonstrated an important difference between the oocytes of

TABLE 1. PREMATURE CHROMOSOME CONDENSATION OF RAT AND MOUSE CUMULUS CELL NUCLEI INJECTED INTO ENUCLEATED RAT AND MOUSE OOCYTES

Origin for			*PCC (%)*	
Cytoplasm	*Nucleus*	*No. of oocytes injected*	+	−
Mouse	Mouse	29	27 (93)	2 (7)
Mouse	Rat	59	53 (90)	6 (10)
Rat	Mouse	21	0 (0)	21 (100)
Rat	Rat	50	0 (0)	50 (100)

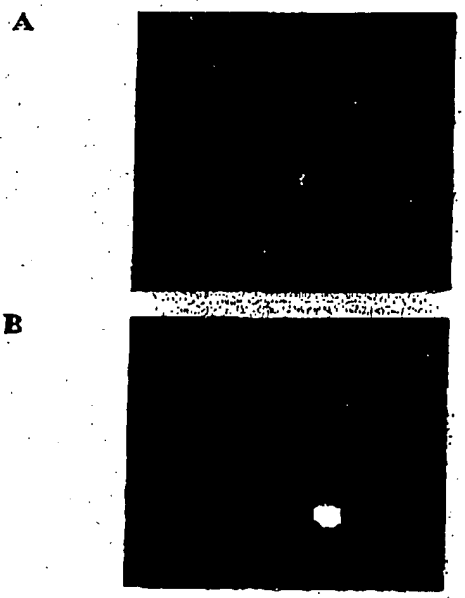

FIG. 1. Hoechst-stained rat cumulus cell nuclei after injection into enucleated oocytes. Premature chromosome condensation is positive in enucleated mouse oocytes (A), but not in enucleated rat oocytes (B).

rats and other mammalian species in their response to enucleation. In the rat, nuclei transferred into an enucleated oocyte did not undergo PCC. This is in contrast to previous reports of PCC in enucleated oocytes of all other mammalian species examined to date. However, nuclei did undergo PCC after transfer to intact rat oocytes. We suggest that MPF activity in rat oocytes is reduced by spontaneous activation or enucleation. This difference between species may be one factor leading to the failure to produce offspring after somatic cell nuclear transplantation in the rat.

The keys t success in the production of cloned mice by the Honolulu method have been considered by the authors (Wakayama et al., 1998; Wakayama et al., 1999; Ogura et al., 2000b); the step includes promoting the PCC of injected nuclei and subsequent PN-like vesicle formation as well as exposing injected nuclei directly to reprogramming factors present in the cytoplasm of recipient oocytes. We postulated that failure to produce cloned rats by the Honolulu method in our previous study (Kato et al., 2001) and others (Hayes et al., 2001; Iannaccone et al., 2001) is due to the unsuitable protocol for PCC induction of the injected nuclei, and that efficient production of PCC in the transferred nucleus is one of the important steps for successful rat cloning. For this purpose, parallel studies on PCC induction in injected cumulus nuclei in intact rat oocytes have optimized the choice of strain (Wistar) and age (4–5 weeks old) of donor rats for host oocytes, and timing of oocyte collection (14 h post hCG injection) and time allowed until nuclear injection (1 h after sacrificing donor), as well as use of neutral cysteine protease inhibitor (20 μg/ml ALLN) (Hirabayashi et al., 2003). It should be noted again that rat oocytes are spontaneously activated during in vitro culture (Keefer and Schuetz, 1982; Zernicka-Goetz, 1991; Kato et al., 2001).

No enucleated rat oocytes were capable of inducing PCC of the rat cell cumulus nuclei after microinjection (Experiment 1). We suggest that the inability of recipient cytoplasm to induce PCC reflects the low level of MPF activity and is independent of the origin of donor cells, because rat cell nuclei underwent PCC in enucleated mouse oocytes and mouse cell nuclei did not undergo PCC in enucleated rat oocytes. There is a

TABLE 2. SERIAL NUCLEAR TRANSFER OF RAT SOMATIC CELLS USING ENUCLEATED MOUSE OOCYTES (1ST NT) AND ENUCLEATED PRONUCLEAR-STAGE RAT ZYGOTES (2ND NT)

Steps	*Parameters*	*Cumulus cell*	*Sertoli cell*
1st NT	No. oocytes injected with nucleus	494	574
	No. oocytes survived injection	340 (69%)	348 (61%)
	No. oocytes forming pseudo-2PN	225 (66%)	195 (56%)
2nd NT	No. couplets prepared	223	195
	No. couplets fused	175 (78%)	185 (95%)
	No. couplets cleaved to 2-cell	103 (59%)	103 (56%)
ET	No. NT eggs transferred	167	174
	No. implantation sites	5 (3%)	2 (1%)
	No. living offspring	0 (0%)	0 (0%)

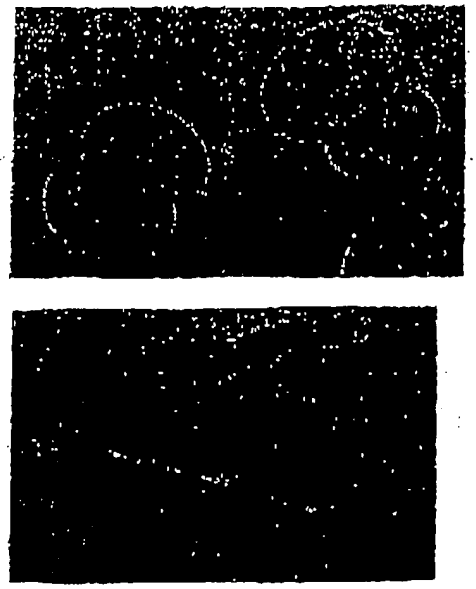

FIG. 2. Two-cell stage rat embryos produced by serial nuclear transfer (A), and the signs for implantation (B).

considerable difference between mouse and rat oocytes in this ability. Ookata et al. (1992) reported that, in starfish oocytes, MPF (Cyclin B/Cdc2 complex) existed near the chromatin structure at the M-phase of the cell cycle although the MPF spread over the cytoplasm at the G2-phase. We also found that PCC of cumulus cell nuclei occurred in rat oocytes from which a part of the cytoplasm was removed (unpublished observation, Hirabayashi et al.). Inactivation of MPF in rat oocytes may be triggered by the spontaneous activation (resumed second meiosis), and be completed by enucleation.

The serial NT procedure (Kwon and Kono, 1996; Ono et al., 2001) is advantageous in the longer exposure time to cytoplasmic reprogramming factors than that during the Honolulu method and in the better support for full-term development of enucleated zygotes, rather than oocytes. The serial NT procedure was also effective in reducing the adverse effect of UV irradiation on the development of NT oocytes (Experiment 3), as the NT oocytes without the replacement of cytoplasm did not develop to the two-cell stage even after 18 h of culture (data not shown). The replacement of cytoplasmic components may have played an essential role in subsequent development of cloned embryos when enucleated mouse oocytes were used as a temporary host for PCC induction of injected rat nuclei (Experiment 2).

Dominko et al. (1999) reported that enucleated bovine oocytes receiving rat skin fibroblast cell nuclei did not develop beyond the four-cell stage, while those receiving sheep fibroblast cell nuclei developed up to Day 39 of pregnancy. Their experiments suggest that the nuclear reprogramming that is necessary for early pregnancy is induced by NT between close species (cattle and sheep). In addition, Wang et al. (2002) reported that neither enucleated mouse oocytes receiving

TABLE 3. SERIAL NUCLEAR TRANSFER OF RAT SOMATIC CELLS USING INTACT RAT OOCYTES (1ST NT) AND ENUCLEATED PRONUCLEAR-STAGE RAT ZYGOTES (2ND NT)

Steps	*Parameters*	*Cumulus cell*	*Sertoli cell*
1st NT	No. oocytes injected with nucleus	576	398
	No. oocytes survived the injection	417 (72%)	292 (73%)
	No. oocytes undergoing PCC	249 (60%)	137 (47%)
	No. oocytes enucleated successfully	207 (83%)	117 (85%)
	No. oocytes survived Sr^{2+} activation	163 (79%)	94 (80%)
	No. oocytes forming pseudo-2PN	28 (17%)	9 (10%)
	No. oocytes forming pseudo-1PN	95 (58%)	52 (55%)
2nd NT	No. zygote-karyoplast couplets	109	61
	No. couplets fused by DC pulse	71 (65%)	47 (77%)
	No. couplets cleaved to 2-cell	25 (35%)	12 (26%)
	No. couplets arrested at 1-cell	44 (62%)	34 (72%)
ET	No. 1-2 cell embryos transferred	69	46
	No. implantation sites	4 (6%)	0 (0%)
	No. living offspring	0 (0%)	0 (0%)

nuclei from 2–4-cell rat embryos nor enucleated rat oocytes receiving nuclei from two-cell mouse embryos cleave in vitro, suggesting the incompatibility between nuclei and cytoplasm of rat and mouse. Failure of reconstructed rat zygotes to develop into offspring after temporary transfer of the nucleus into a mouse oocyte (Experiment 2) may be explained by the contamination of mouse mitochondrial DNA and/or mouse nuclear proteins such as histone during the chromosome formation of rat nuclei.

In conclusion, reconstructed rat zygotes can be prepared by serial NT procedures, but developmental competence of the zygotes remains to be clarified. Efficiency of producing reconstructed rat embryos in Experiment 2 using enucleated mouse oocytes is higher than that in Experiment 3 using intact rat oocytes, but there may be an adverse effect on implantation.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 12794020). We thank Dr. A. Ogura of RIKEN Bio-Resource Center, Tsukuba, Japan, for helpful suggestions, and T. Aoto and M. Terauchi of YS New Technology Institute, Inc., Tochigi, Japan, for technical assistance.

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Development of fertilized embryos transferred to oviducts of immature mice.

Papaioannou VE, Ebert KM.

The in-vitro culture of fertilized 1-cell mouse embryos to the blastocyst stage is associated with subsequent decreased viability. In this study, 1-cell embryos were cultured for 3 days in the reproductive tract of immature female mice as an alternative to in-vitro culture. Embryos that spent 3 days in immature females were developmentally more advanced, had higher cell numbers and better viability, as measured by development to mid-gestation, after transfer to pseudopregnant recipient females than did embryos maintained for the same period in culture. Embryos that developed in immature females had lower cell numbers but comparable rates of development and subsequent viability when compared with embryos transferred to synchronous pseudopregnant females for the same preimplantation period. The immature mouse oviduct is therefore a suitable alternative environment to in-vitro culture or a pseudopregnant host for complete preimplantation development and has the additional advantage that synchrony between embryo and temporary host is not necessary. This method will allow for evaluation of manipulation procedures while maintaining viability before the embryos are finally committed to a foster mother for development to term.

PMID: 3701702 [PubMed - indexed for MEDLINE]

BIOLOGY OF REPRODUCTION 62, 1270–1277 (2000)

The Proteasome Is Involved in the First Metaphase-to-Anaphase Transition of Meiosis in Rat Oocytes[1]

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ABSTRACT

The proteasome engages in protein degradation as a regulatory process in biological transactions. Among other cellular processes, the proteasome participates in degradation of ubiquinated cyclins in mitosis. However, its role in meiosis has not been established. Resumption of meiosis in the oocyte involves the activation of maturation promoting factor (MPF), a complex of p34cdc2 and cyclin B. Inactivation of this factor, occurring between the two meiotic divisions, is associated with degradation of cyclin B. In this study, we examined the possible involvement of the proteasome in regulation of the exit from metaphase I in spontaneously maturing rat oocytes. We found that upon resumption of meiosis, proteasomes translocate to the spindle apparatus. We further demonstrated that specific inhibitors of proteasome catalytic activity, MG132 and lactacystin, blocked polar body extrusion. Chromosome and microtubule fluorescent staining verified that MG132-treated oocytes were arrested at metaphase I. Intervention of proteasomal action with this inhibitor also resulted in accumulation of cyclin B and elevated activity of MPF. These data demonstrate that proteasomal catalytic activity is absolutely essential for the decrease in MPF activity and completion of the first meiotic division. Its translocation to the spindle apparatus may facilitate the timely degradation of cyclin B.

INTRODUCTION

Meiosis in mammalian oocytes starts during embryonic life and arrests around birth at the first prophase. Meiosis resumes in the postpubertal mammalian female, when a selected number of oocytes at each sexual cycle mature into fertilizable eggs [1]. Reinitiation of meiosis is manifested by chromosome condensation, disintegration of the nuclear envelope (germinal vesicle breakdown [GVBD]), and spindle formation. The first meiotic division concludes by segregation of the homologous chromosomes and emission of the first polar body (PB). Immediately thereafter, the second meiotic division commences and is arrested again at the second metaphase (MII). Meiosis terminates upon fertilization, when sister chromatids are segregated and the second PB is extruded [2].

Oocyte maturation is triggered in vivo by a surge of the pituitary gonadotropin LH, which uncouples the oocyte from the surrounding cells of the ovarian follicle. This uncoupling results in the decrease of cAMP concentrations in the oocyte, which is a prerequisite for meiosis resumption [3]. A similar reduction in intraoocyte content of cAMP, consequent to their separation from the enveloping ovarian follicle, results in spontaneous maturation [4].

Reinitiation of meiosis represents the transition from G2 to M phase of the cell cycle and is regulated by the maturation promoting factor (MPF), a complex of the cyclin-dependent kinase, p34cdc2, and cyclin B (reviewed in [2]). Cyclin binding to p34cdc2 forms the pre-MPF complex, the activation of which is achieved by dephosphorylation of Thr-14 and Tyr-15 on p34cdc2 [5]. MPF inactivation, occurring between the two meiotic divisions, is associated with degradation of cyclin B1 [6].

Several mechanisms ensure the precise action of key regulators in the complex process of meiotic division, in which meticulous regulation prevails. Phosphorylation/dephosphorylation reactions represent a well-known mechanism for modifying protein activity. Alongside this posttranslational modification, protein degradation has been shown to participate in activation and inactivation of several signal transduction pathways [7]. One of the emerging representatives of this notion is the proteasome, which is the primary participant in the mechanism of protein degradation.

The proteasome is a multicatalytic protease that is able to hydrolyze C-terminal peptide bonds to acidic, basic, and hydrophobic amino-acid residues. It comprises approximately 1% of the protein in mammalian cells and serves as the main cellular protein degradation pathway [8, 9]. Electron microscopy of purified proteasomes from *Xenopus* oocytes reveals a large (26S) complex structure of a dumbbell shape [10], consisting of a central core catalytic subunit (20S) shaped like a cylinder, bordered by two large components (19S) at the ends of the core. Lactacystin, a *Streptomyces* metabolite, was found to inhibit proteasomal proteolysis by binding and modifying its catalytic subunit [11–13].

The idea that the proteasome participates in regulation of meiosis was initially based on reports in lower-order eukaryotes, in which changes in its catalytic activity during oocyte maturation were demonstrated [14–19]. Prevention of GVBD by inhibition of proteasomal proteolytic activity further suggested that this proteinase is involved in reinitiation of meiosis in toad and starfish oocytes [17, 19]. Other studies suggested the involvement of proteasomal action in termination of meiosis [20]. Furthermore, the establishment of the elaborate enzymatic system that leads to ubiquitin-mediated proteolysis of cyclin B [21–23] was characterized in clam oocytes entering the first meiotic division [24]. Nevertheless, the precise action of the proteasome at the transition between the two meiotic divisions has not been demonstrated. Furthermore, the involvement of the proteasome during meiosis of mammals is unknown.

[1]This work was partially supported by a grant from the Maria Zondek Hormone Research Fund (N.D.) and the Leo and Julia Forchheimer Center for Molecular Genetics of the Weizmann Institute of Science (A.A.). A.A. is the incumbent of the Joyce and Ben B. Eisenberg professional chair of Molecular Endocrinology and Cancer Research. N.D. is the incumbent of the Phillip M. Klutznick professorial chair of Developmental Biology.
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Received: 17 September 1999.
First decision: 19 October 1999.
Accepted: 29 December 1999.

ISSN: 0006-3363. http://www.biolreprod.org

The general goal of our study was to elucidate the role of the proteasome during mammalian oocyte maturation. Our research was specifically directed at the first round of meiosis, a unique case of cell division that does not involve separation of sister chromatids but rather segregation of homologous chromosomes. We assumed that information generated by the many studies on mitosis, or even on the second round of meiosis, is not necessarily applicable to this particular example of the cell cycle. We herein report that during resumption of meiosis in rat oocytes, the proteasomes translocate to the spindle apparatus. Inhibition of the catalytic activity of the proteasome results in cyclin B accumulation, which maintains MPF activity and arrests the oocyte at metaphase I (MI) by preventing extrusion of the first PB.

MATERIALS AND METHODS

Reagents and Antibodies

Leibovitz's L-15 tissue culture medium was purchased from Gibco BRL (Paisley, Scotland). Antibiotics were purchased from Bio-Lab Ltd. (Jerusalem, Israel). MG132 (Z-leu-leu-CHO) and lactacystin were purchased from Calbiochem (La Jolla, CA). A calpain II inhibitor, LLmL (*N*-acetyl-L-leu-leu-normethioninal), leupeptin, isobutylmethylxanthine (IBMX), histone H1 (type III-S), monoclonal mouse anti-α and β-tubulin antibodies, fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibodies, DAPI (4′,6′-diamidino-2-phenylindole), and fetal bovine serum were purchased from Sigma (St. Louis, MO). Cy3-conjugated anti-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies were purchased from Zymed (San Francisco, CA). Protein A-HRP, [γ-^{32}P]adenosine 5′-triphosphate (3000 Ci/mmol), and enhanced chemiluminescence (ECL) Western blotting detection reagents were purchased from Amersham (Buckinghamshire, England). Monoclonal mouse anti-cyclin B1 antibodies [25] were a kind gift of Dr. M. Brandeis (The Hebrew University, Jerusalem, Israel). The antibody was raised against residues 160–300 of hamster cyclin B1, which is 99.2% identical to rat cyclin B1 and shares no homology with other rat cyclins. Antisera against rat granulosa 20S proteasomes were raised in our laboratory [26].

Animals

Sexually immature female Wistar rats (23–25 days old) from our departmental colony received s.c. injections of 15 IU of eCG (Sanofi Sante Nutrition Animale, Libourne, France) in 0.1 ml of 0.9% NaCl for induction of follicular development. The rats were killed by cervical dislocation 48 h later. The investigation was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy of Science, Bethesda, MD)

Oocyte Recovery and Culture

Oocytes were recovered and incubated as described previously [27]. Briefly, the oocytes were isolated into Leibovitz's L-15 tissue culture medium, supplemented with 5% fetal bovine serum, 100 IU/ml penicillin, 50 μg/ml streptomycin, and 0.25 μg/ml fungizone. Cumulus cells were removed enzymatically by collagenase (50 IU/ml, 30 min), and denuded oocytes were incubated in a 37°C humidified incubator. At the end of incubation, the oocytes were analyzed for maturation by differential interference contrast (DIC) microscopy. The presence of a germinal vesicle (GV) was used to classify oocytes as meiotically arrested. Resumption of meiosis, which was indicated by GVBD, occurred spontaneously in oocytes incubated for 4 h after their isolation from ovarian follicles. To prevent spontaneous GVBD, oocytes were incubated with the cAMP phosphodiesterase inhibitor, IBMX [28]. The first PB was emitted at 10–12 h of incubation in inhibitor-free medium, and after an overnight incubation, the oocytes were arrested at MII. For proteasomal inhibition, oocytes were released and further incubated in medium containing the proteasome inhibitors MG132 and lactacystin.

For transient exposure, the oocytes were recovered and placed into inhibitor-containing medium. After 24-h incubation, the oocytes were either placed into fresh inhibitor (continuous exposure) or washed 5 times and further incubated in inhibitor-free medium (transient exposure). The oocytes were examined morphologically after an additional 12 h.

Western Blot Analysis and Cytochemistry

At the end of the specified incubation time, the oocytes were lysed in lysis buffer (1% Triton X-100, 50 mM Hepes pH 7.2, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium-orthovanadate, 10% glycerol, 30 mM NaF, 30 mM sodium-pyrophosphate) and subjected to Western blot analysis as described previously [29]. The following antibodies were used: rabbit antisera against rat granulosa 20S proteasomes (1:1000 dilution), monoclonal mouse anti-β-tubulin antibodies (1:1000 dilution), and monoclonal mouse anti-cyclin B1 antibodies (1:750 dilution). The relevant HRP-conjugated secondary antibodies were used, and immunoreactive bands were detected by ECL. Densitometric analysis was performed using the 420oe densitometer (Pdi, Huntington Station, NY) supported by Quantity One software (Pdi).

For immunofluorescence, oocytes were fixed and immunostained with rabbit antisera against rat granulosa 20S proteasomes (1:200 dilution), incubated with the secondary Cy3-conjugated anti-rabbit antibodies (1:250 dilution) and monoclonal mouse anti-β-tubulin antibodies (1:200 dilution), and then incubated with the secondary FITC-conjugated rabbit anti-mouse antibodies (1:200 dilution) as described previously [29]. DAPI was added along with the relevant secondary antibody (1:200 dilution), or by itself. Oocytes in 50% glycerol/PBS were mounted on silicon-coated glass slides and covered by coverslips resting on a silicone ring containing 100 μm glass beads that served as spacers. The oocytes were visualized by both phase-contrast and fluorescent microscopy, using an Optiphot-2 microscope (Nikon Co., Tokyo, Japan) equipped with BP546/455 filters. Alternatively, a laser scanning confocal microscope (Zeiss, Oberkochen, Germany; LM410) was used.

H1 Kinase Activity

Histone H1 kinase activity was measured in lysates of 25 oocytes, prepared by freezing and thawing in 10 μl kinase buffer (15 mM 3-(*N*-morpholino)propanesulfonic acid [MOPS], 80 mM β-glycerophosphate, 10 mM EGTA, 15 mM $MgCl_2$, 0.1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml PKI, a cAMP-dependent protein kinase inhibitor peptide). Kinase reactions were initiated by the

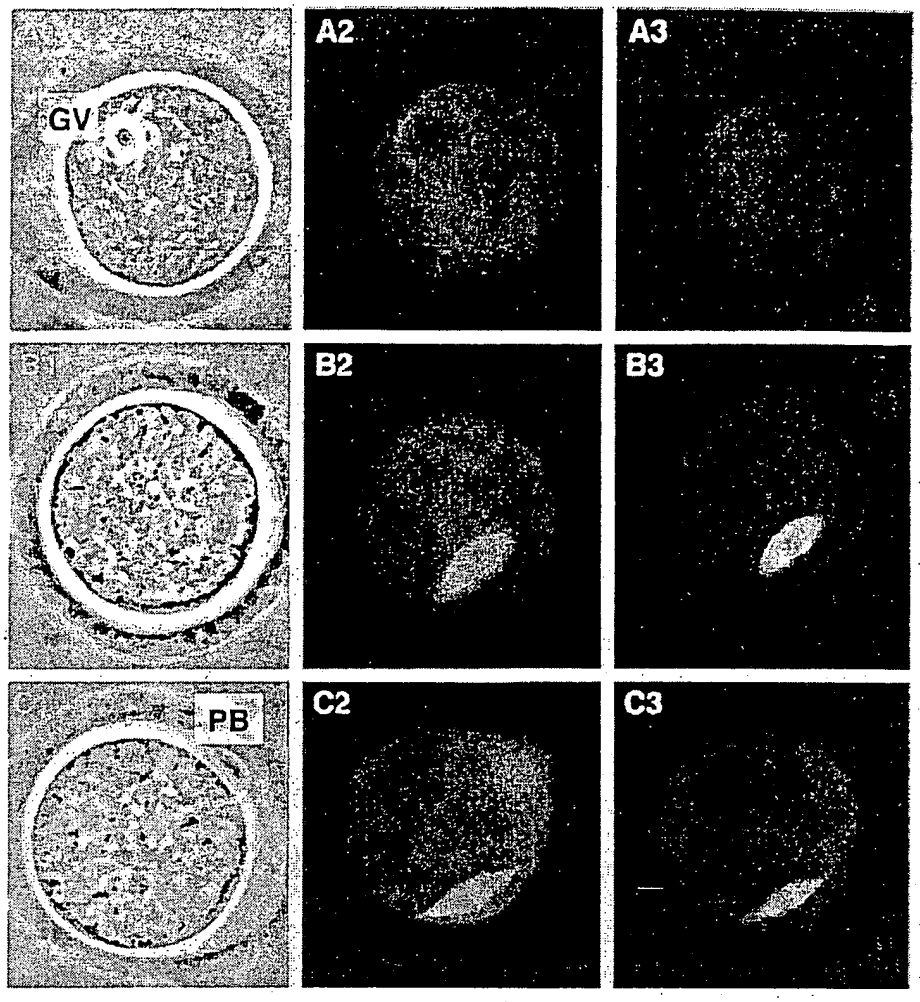

FIG. 1. Localization of proteasomes in rat oocytes during meiosis. Spontaneously maturing rat oocytes were fixed at various stages of meiosis and were immunostained with anti-20S proteasome antibodies (red) and β-tubulin (fluorescent yellow-green). A) A meiotically arrested GV oocyte, incubated in the presence of 0.2 mM IBMX, that has not yet assembled the spindle apparatus (A2), showing low concentration of proteasomes (A3) in the ooplasm and a relatively high perinuclear concentration around the GV. B) An oocyte, 8 h after isolation from ovarian follicle, resuming meiosis in MI, displaying translocation of the proteasome (B3) to the spindle apparatus (B2). C) A mature oocyte, after an overnight incubation, arrested at MII, displaying translocation of the proteasomes (C3) to the spindle apparatus (C2); note a low labeling associated with the first PB.

addition of 10 μl of substrate buffer (2 mg/ml histone H1, 2 mM dithiothreitol (DTT), 5 μCi [γ-^{32}P]ATP), and the reactions were carried out at 30°C for 30 min. Kinase reaction products were subjected to SDS-PAGE and autoradiography. Densitometric analysis was performed utilizing the Fujix BAS1000 phosphoimager, supported by MacBas software (Fujix, Tokyo, Japan).

RESULTS

Localization of the Proteasome in Rat Oocytes During Meiosis

Possible changes in proteasome localization during resumption of meiosis were examined by double-staining of spontaneously maturing rat oocytes with antibodies against 20S proteasomes as well as against β-tubulin. We found that in meiotically arrested oocytes the proteasomes are localized at the perinuclear region (Fig. 1, A3). Oocytes resuming meiosis exhibited low cytoplasmic concentration of the proteasomes and their clear translocation to the spindle apparatus of the first meiotic division (Fig. 1, B1–B3). Oocytes arrested at MII (Fig. 1C) exhibited as well an unequivocal colocalization of the proteasome with the MII spindle apparatus (Fig. 1, C2–C3). Some staining of the proteasome was also associated with the first PB (Fig. 1, C3).

The pattern of proteasome expression during meiotic division was examined by Western blot analysis in oocytes extracted at various stages of meiosis. Two major bands in the range of M_r 25–35 × 10^{-3}, which correspond to proteasomal subunits [29], were detected in the oocyte lysates (Fig. 2). A slight increase in the protein amount was observed upon progression from GV to GVBD. No further change in the proteasome concentration was evident in oocytes proceeding to MII.

Effect of Proteasome Inhibitors on Resumption of Meiosis

In order to characterize the function of the proteasome in meiosis, selective inhibitors of its catalytic activity were analyzed for their effect on spontaneously maturing oocytes. The oocytes were monitored morphologically for GVBD and PB extrusion.

As shown in Figure 3A, lactacystin, a potent and highly specific irreversible inhibitor of proteasomal proteolytic activity, and MG132, a potent reversible proteasome inhibitor, blocked PB extrusion at a concentration range of 1–10 μM. Inhibitors of other proteolytic pathways such as the calpain II inhibitor LLmL (Fig. 3A), as well as leupeptin, an inhibitor of lysosomal degradation (data not shown) did not inhibit PB extrusion even at a high concentration of 50 μM. Interestingly, none of the inhibitors employed in this set of experiments interfered with GVBD. Figure 3B depicts the results of testing the reversibility of the inhibition by lactacystin and MG132. Oocytes incubated for 24 h with these agents were extensively washed with control media. Further incubation was performed for 12 additional hours in the presence or absence of the inhibitors (continuous exposure

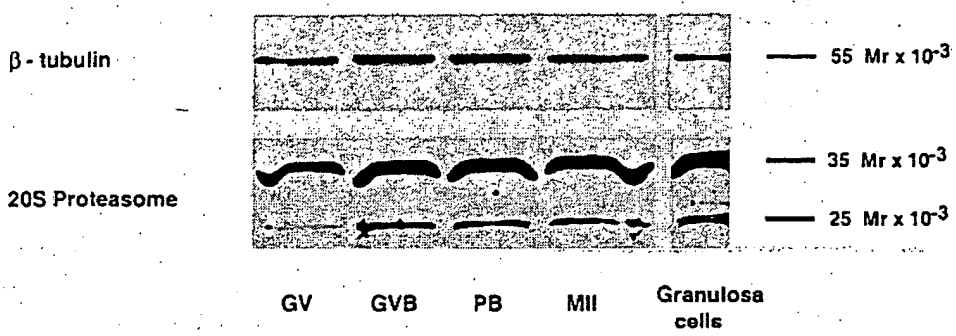

FIG. 2. The expression pattern of proteasomes during meiosis. Spontaneously maturing rat oocytes (250 per lane) were extracted at various stages of meiosis as follows: GV, incubated in the presence of 0.2 mM IBMX, GVBD (3–4 h after isolation), PB (10–12 h after isolation), and MII (after overnight incubation). The right lane contains an equivalent amount of protein extracted from rat granulosa cells. The extracts were separated and immunolabeled with anti-20S proteasome antibodies. Anti-β-tubulin antibodies were used in order to normalize for protein concentration. The experiment was repeated 4 times; the results of one representative experiment are presented.

and transient exposure, respectively). The inhibition of PB extrusion by MG132 was reversible whereas that induced by lactacystin was not (Fig. 3B).

The morphology of the arrested oocytes presented a particular interest. Oocytes incubated in inhibitor-free medium extruded the PB as expected, 10 h after their isolation (Fig. 4A). In contrast, the MG132-treated oocytes that failed to extrude the PB exhibited an elongated protrusion (Fig. 4B), which was evident throughout the incubation of the oocytes with the drug.

In order to identify the time during meiosis at which the proteasome-sensitive event takes place, oocytes were isolated in inhibitor-free medium, and MG132 was added at different times during incubation. We found that addition of MG132 at any time, even just before PB extrusion, effectively prevented its emission (data not shown). The precise stage of inhibition throughout meiosis was confirmed by chromosome staining. Control, MII-arrested oocytes incubated in inhibitor-free medium for 24 h showed two stained DNA aggregates, indicating segregation of chromosomes between the oocyte and PB (Fig. 5A′). On the other hand, the MG132-treated oocytes exhibited only one stained aggregate localized in the elongated protrusion, suggesting failure of chromosome segregation and incomplete metaphase-to-anaphase transition of MI (Fig. 5B′).

Double staining of chromosomes and microtubules corroborated that oocytes were experimentally arrested at the first metaphase-to-anaphase transition. Whereas control oocytes presented chromatids arranged on the second metaphase spindle (Fig. 6A) with the remnants of their homologues in the PB (Fig. 6A′), MG132-treated oocytes showed incomplete segregation of the chromosomes on the first meiotic spindle (Fig. 6B). Chromosomes that were partially segregated but still localized on the metaphase plate were also observed in many oocytes examined by conventional microscopy. Nevertheless, anaphase was never observed.

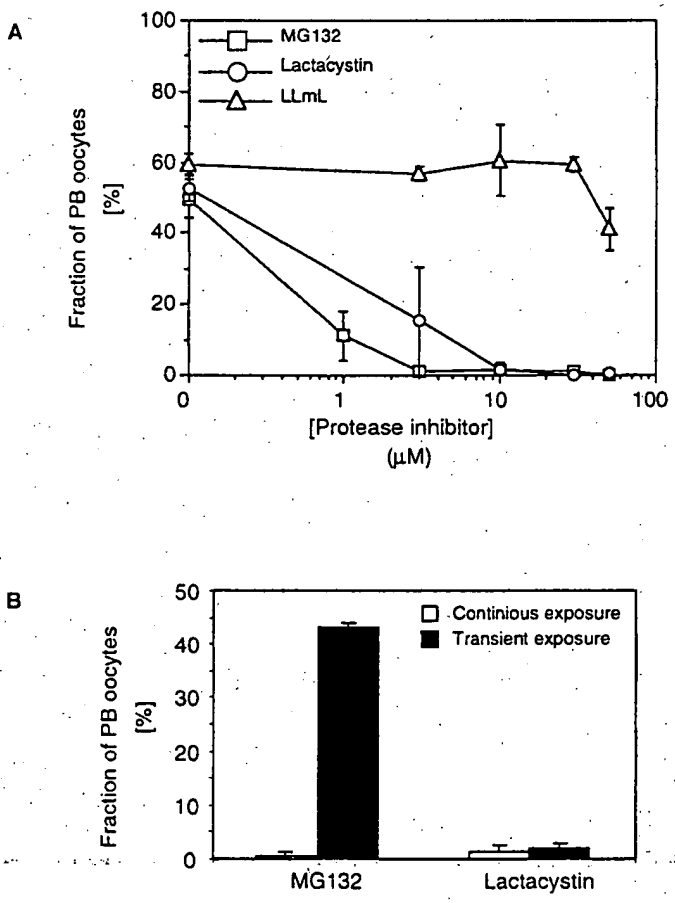

FIG. 3. A) The effect of proteasomal activity inhibition on PB extrusion. Rat oocytes were incubated for 24 h with MG132 (squares), lactacystin (circles), and LLmL (triangles). The fraction of oocytes that extruded the first PB is presented. The means of at least 3 different experiments (minimum of 160 oocytes for each experimental point) are presented along with their standard errors. B) Recovery from the effect of the proteasome inhibitors. Oocytes incubated for 24 h in 10 μM of either MG132 or lactacystin were extensively washed and further incubated for 12 h in the presence (continuous exposure) or absence (transient exposure) of the inhibitor. The fraction of oocytes that extruded a PB is presented. Means of at least 3 different experiments (minimum of 160 oocytes for each experimental point) are presented along with their standard errors.

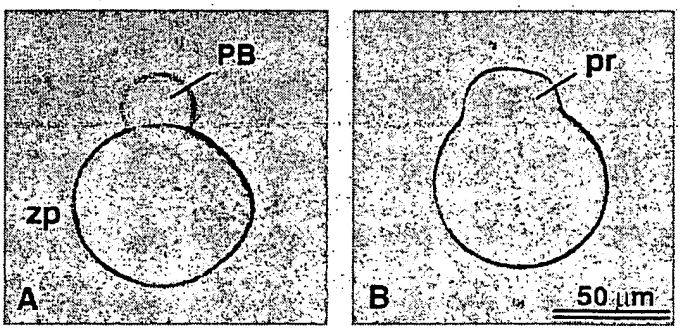

FIG. 4. Morphology of MG132-arrested oocytes. Rat oocytes were incubated in the absence (A) or presence (B) of MG132 and were monitored with DIC microscopy. A) First PB extrusion in control oocytes incubated for 24 h. zp, Zona pellucida. B) Inhibition of PB extrusion by MG132; note the elongated protrusion (pr).

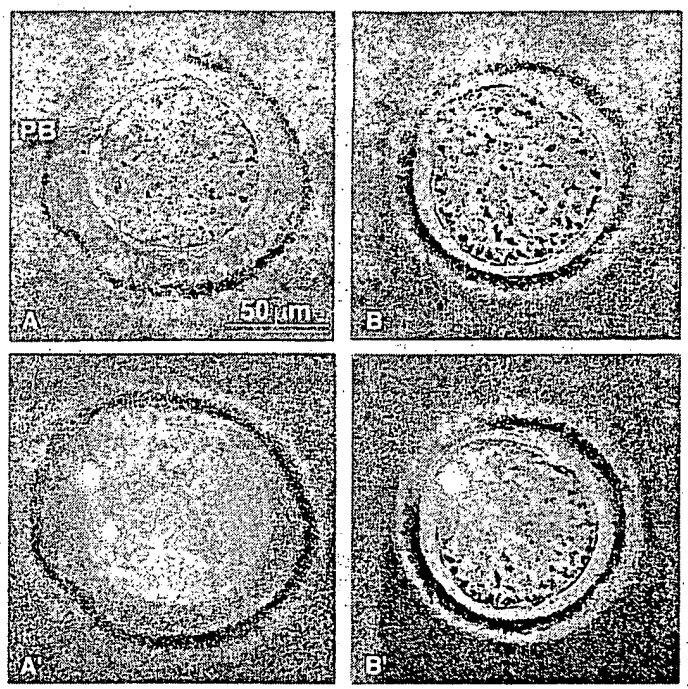

FIG. 5. Cellular localization of DNA in MG132-arrested oocytes. Rat oocytes incubated in the absence (**A, A'**) or presence (**B, B'**) of MG132 were fixed and stained for DNA (DAPI staining) and examined with phase contrast (**upper**) or fluorescent microscopy (**lower**). **A**) Extrusion of the first PB in an oocyte incubated in inhibitor-free medium for 24 h. **A'**) Note segregation of chromosomes between the oocyte and PB. **B**) MI-arrested oocyte after 24 h incubation with MG132 (30 µM). **B'**) Note failure of chromosomes to segregate.

Degradation of Cyclin B by Proteasomes

The fact that exit from first meiosis was prevented by the proteasome inhibitors suggested that cyclin B1 could be a likely candidate for proteasome degradation. To test this hypothesis, we subjected extracts of control and MG132-treated oocytes to Western blot analysis using anti-cyclin B1 antibodies. Figure 7 depicts a relatively small amount of cyclin B1 in control oocytes that extruded the first PB after 10 h of incubation, reflecting the degradation of cyclin B1 between the two meiotic divisions. MG132-treated oocytes at 10-h incubation time expressed higher amounts of this protein (Fig. 7, upper panel). Densitometric analysis disclosed a 2.9-fold increase in the amount of this protein in MG132-treated oocytes compared to controls after 10 h of incubation (Fig. 7, lower panel). Interestingly, during the second meiotic division, in which cyclin B1 is synthesized preceding the second metaphase, the same pattern of cyclin accumulation occurred. Oocytes that were incubated with MG132 for 24 h accumulated more cyclin B1 than control, MII-arrested oocytes. In a further experiment, we found that the amount of cyclin B1 was lower in oocytes recovering from MG132 inhibition than in oocytes continuously exposed to this inhibitor (data not shown). No changes were observed in the amount of tubulin in the oocytes incubated under the same conditions.

The accumulation of cyclin B was corroborated by measurements of MPF activity in the presence or absence of MG132. MPF was monitored in oocytes resuming meiosis by a histone H1 kinase assay—an assay that is routinely used for monitoring p34cdc2 kinase activity. Analysis of H1 kinase activity, under conditions of proteolytic inhibition, revealed that the addition of MG132 resulted in a relatively higher kinase activity at the time of first PB extrusion. In control oocytes that had just extruded a PB (10 h after isolation), the amount of phosphorylated histone H1 was relatively low, representing the inactivation of MPF between the two meiotic divisions, due to cyclin B degradation (Fig. 8). Inhibition of proteolytic activity in oocytes by incubation in MG132 for 10 h resulted in a significant, 2.8-fold increase in MPF activity (Fig. 8, lower panel). An increase in the amount of phosphorylated histone H1 under conditions of proteolytic inhibition was also evident at the second meiotic division. The addition of MG132 resulted in a relatively higher kinase activity in the treated oocytes as compared to control, MII-arrested oocytes at 24-h incubation time.

DISCUSSION

In this study, we examined the role of protein degradation by the proteasome in rat oocytes resuming meiosis. Our study provides the first demonstration that inhibition of the catalytic activity of the proteasome arrests the oocyte at MI, preventing PB extrusion. It further shows that these MI-arrested oocytes accumulate cyclin B and maintain a high level of MPF activity. It also reveals that throughout resumption of meiosis, proteasomes translocate to the spindle apparatus. Our study strongly suggests that proteasomal

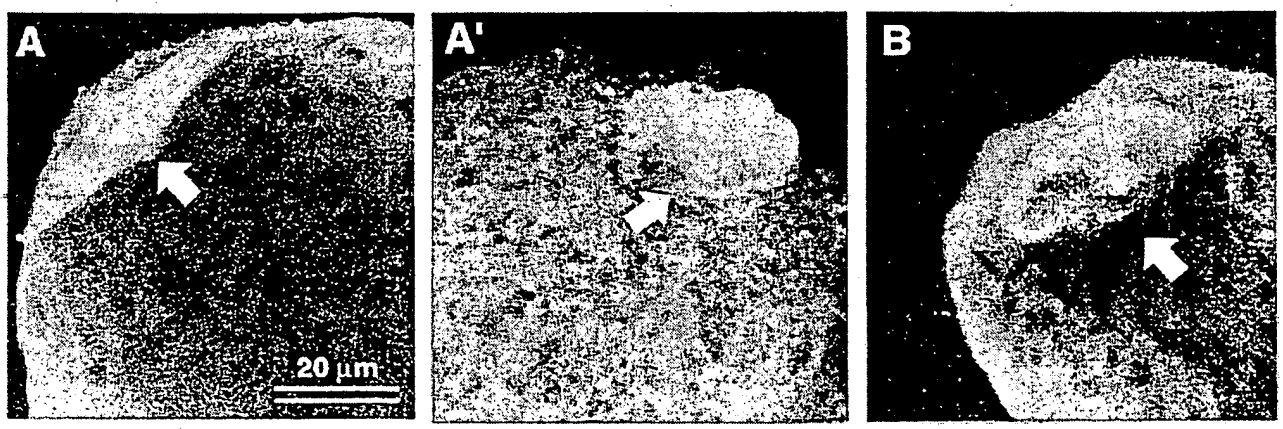

FIG. 6. Cellular localization of DNA and microtubules in MG132-arrested oocytes. Images of spindles from oocytes incubated in the absence (**A, A'**) or presence (**B**) of MG132 (10 µM) for 24 h, double-stained for β-tubulin (fluorescent green) and DNA (blue), and examined with confocal microscopy. **A**) The chromatids are arranged at the MII plate, in a longitudinal section of the spindle. **A'**) High concentration of tubulin in the PB with the remnants of the homologous chromosomes. **B**) Chromosomes arrested in metaphase/anaphase I; a diagonal section of the spindle.

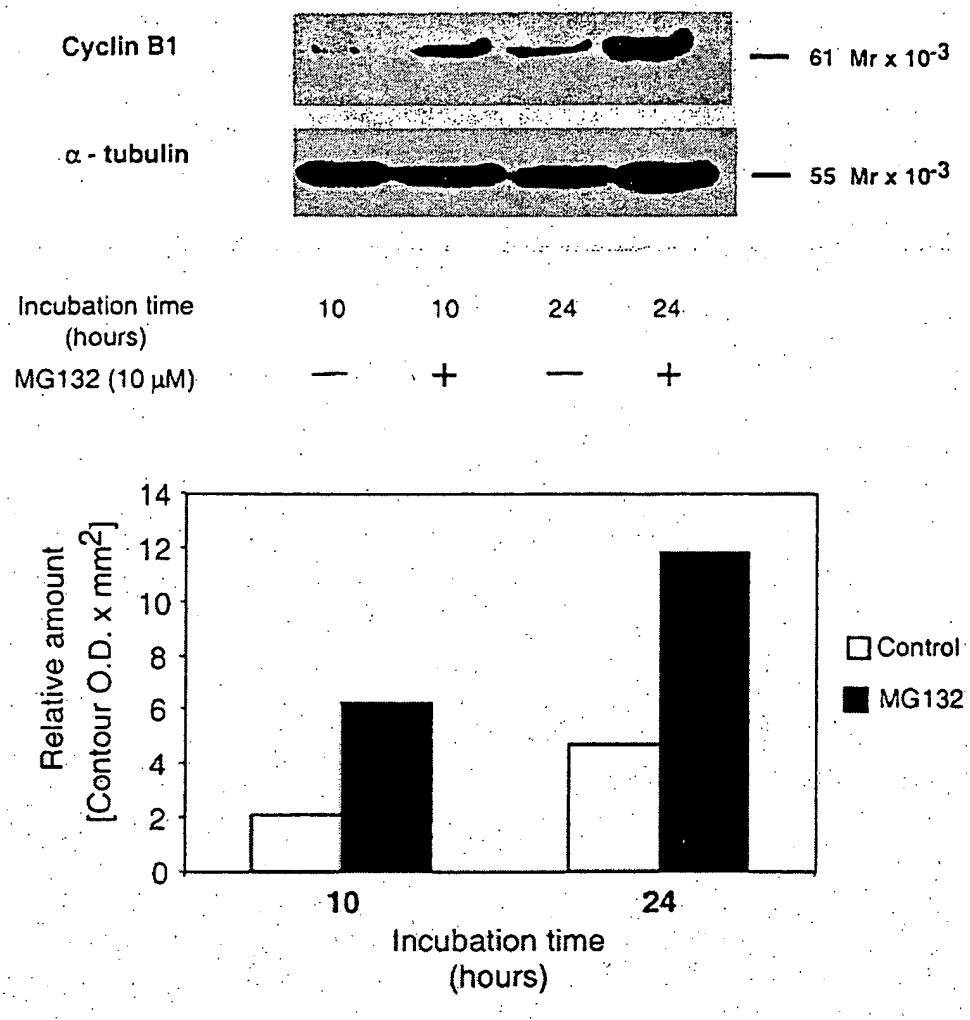

FIG. 7. The effect of MG132 on cyclin B1 level of expression. Spontaneously maturing rat oocytes (250 per lane) were extracted at the indicated times of incubation in the presence or absence of MG132 (10 μM). The extracts were separated and immunolabeled using anti-cyclin B1 and α-tubulin antibodies. The experiment was repeated 5 times. **Upper)** Results of one representative experiment; **lower)** the densitometric analysis of this experiment.

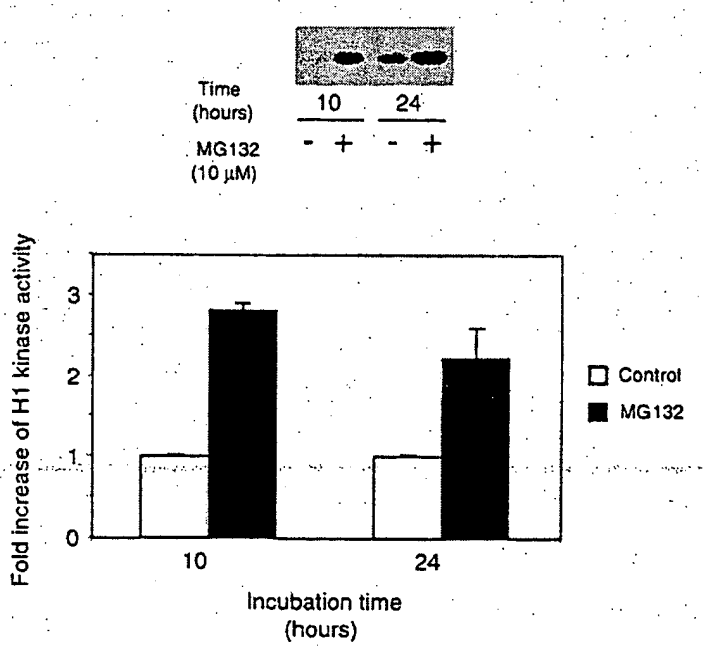

FIG. 8. The effect of MG132 on H1 kinase activity during maturation of rat oocytes. Spontaneously maturing rat oocytes (25 per lane) were extracted at the indicated times of incubation in the presence or absence of MG132 (10 μM) and assayed for H1 kinase activity. **Upper)** Results of one representative experiment; **lower)** means and SE of pooled results from 3 individual experiments.

activity is critical for completion of meiosis in rat oocytes. Furthermore, its localization around the spindle apparatus could facilitate degradation of the relevant cellular substrate.

The spindle assembly checkpoint is a part of the surveillance mechanism that monitors the completion of critical cell cycle events and allows the subsequent cell cycle transition to occur [30–32]. The translocation of proteasomes to the spindle apparatus strongly suggests that proteolysis serves as an effector of spindle function in meiotic division. This possibility is further strengthened by our finding that inhibitors of proteasomal catalytic activity arrest the oocytes at MI, preventing the completion of the first round of meiosis. In addition, the ability of proteasome inhibitors to block PB extrusion at any time point suggests that the proteasome-sensitive event occurs shortly before the metaphase/anaphase transition. This conclusion is supported further by our observation that MG132-treated oocytes exhibited an elongated protrusion, reflecting their unsuccessful "effort" to emit a PB. Therefore, the MI arrest implies that, at least in the rat, proteasomes degrade proteins that are crucial for anaphase to occur. Taken together, these results point towards completion of the first round of meiosis as a specific cellular event dependent on proteolysis.

Interestingly, in the rat, in contrast to lower eukaryotes [17, 19], none of the proteolytic inhibitors affected early meiotic events, such as GVBD. This inconsistency can be added to other differences between meiosis of rat oocytes

and those of lower organisms. One such difference is manifested at the very early stage of meiosis reinitiation. Rat oocytes resume meiosis spontaneously upon their separation from the follicle [33], whereas in lower organisms an external stimulatory ligand (progesterone, 1-methyladenine, or sperm) is absolutely necessary for their exit from G2-arrest.

Earlier studies have demonstrated the proteasomal destruction of mitotic cyclins [34–36], which is mediated via the ubiquitin pathway [21–23]. Direct evidence of proteasomal digestion of cyclin B was only recently presented in a cell-free system. Tokumoto et al. [20] reported that purified 26S proteasomes were shown to cleave recombinant cyclin B1 of goldfish and *Xenopus* oocyte extracts. However, that study failed to demonstrate that cyclin B cleavage affected MPF kinase activity. Our study shows for the first time a proteasome-dependent degradation of cyclin B in intact oocytes that clearly correlates with a decreased activity of MPF.

Similar to our findings in meiosis, the timely degradation of cyclin B1 was shown to be necessary for exit from mitotic M phase [21]. However, nondegradable mutants of cyclin B1 did allow separation of sister chromatids, arresting the cell cycle in telophase rather than anaphase [37–39]. These studies suggest that proteolysis of proteins other than cyclin B1 could be the direct cause of metaphase-to-anaphase transition [32]. The other candidate proteins for degradation by the ubiquitin-dependent pathway are INCENP [40] and CLiP [41], which are positioned between sister chromatids before anaphase [42]. Mammalian homologues of yeast PDS1 and CUT2, both of which are degraded during anaphase and arrest sister chromatid segregation in their nondegradable form, could also serve as candidate proteins for proteasomal action at the completion of cell division [43]. Indeed, very recently such a protein, vSecurin, was identified in vertebrates and found to be degraded by the proteasome [44]. A nondegradable mutant form of this protein blocked sister chromatid separation in *Xenopus* cycling egg extracts [44]. However, as mentioned previously, unlike the case in mitosis and the second round of meiosis, completion of the first meiotic division involves separation of homologous chromosomes. In this unique case of cell division; sister chromatids should be held together. Therefore, to avoid aneuploidy, the above-mentioned proteins should be protected from degradation and cannot serve as candidates for proteasomal degradation activity. Whether other as yet unidentified proteins are destroyed at the metaphase-to-anaphase transition of the first meiotic division of the oocyte, allowing segregation of the homologous chromosomes, should be examined.

ACKNOWLEDGMENTS

We would like to thank Profs. Alvin Kaye Chaim Kahana and Geula Gibori for critical reading of the manuscript. The anti-cyclin B1 antibodies were kindly provided by Dr. Michael Brandeis.

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